Escola Tècnica Superior
d'Enginyeria Agrària de Lleida

SIMPOSI SOBRE EL DECLIVI DE LES POBLACIONS D'AMFIBIS

SIMPOSIO SOBRE EL DECLIVE DE LAS POBLACIONES DE ANFIBIOS

SYMPOSIUM SUR LA DIMINUTION DES POPULATIONS AMPHIBIENS

SYMPOSIUM ON THE DECLINING OF THE POPULATIONS OF AMPHIBIANS

Llibre de resums

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Knapp, R. & Matthews, K., 2000. Non-native fish introductions and the decline of the mountain yellow-legged frog from within protected areas. *Conservation Biology* 14 (2): 428-438.

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IRIDOVIRUS-LIKE PARTICLES IN LESIONS OF DISEASED *Triturus marmoratus* FROM CARRIS AND BATATEIRO

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INTRODUCTION

The family Iridoviridae encompasses large icosahedral DNA viruses that assemble in the cytoplasm of the infected cells. Their genomes are terminaly redundant and circularly permuted (Williams, 1996). African swine fever virus, the single member of the Asfaviridae family, resembles morphologically the Iridoviridae, but the structure of its genome is different, resembling the Poxviridae (Anonymous, 2003; González, 1986).
Many other uncharacterised viruses resemble the Iridoviridae. These are usually found by transmition electron microscopic examination of pathological specimens (ex: Cunningham et al., 1996). Their final classification as Iridoviridae must wait their molecular biological characterization (Williams, 1996).

The first vertebrate iridoviruses were found in normal frogs while searching for the aetiological agent of the Lucké frog kidney tumor, that turned out to be an herpesvirus (Granoff, 1989). Although experimental evidence indicated that these viruses could produce disease in susceptible hosts, they remained for a long time considered as non pathogenic and almost a biological curiosity (Hess, 1981).

Outbreaks of highly lethal infections in aquaculture facilities around the world begun to be detected in the late eighties, and were found to be caused by iridoviruses of the ranavirus genus (Ahne et al., 1998). Infection of wild fish with these viruses was also detected with increased frequency, and pathogenicity of the same viruses to frogs was demonstrated, implicating the herpetofauna as reservoirs of viruses pathogenic for fish and vice-versa (Moody & Owens, 1994; Ahne et al., 1995). Evidence for widespread lethal ranavirus infections of amphibians and also reptiles came later (Cunningham et al, 1996; Mao et al., 1997).

These observations show that the Iridoviridae are highly pathogenic for susceptible populations. Translocation and introduction of animals to new geographic regions as a result of human activities is thought to promote the spreading of infections among amphibian populations (Daszak et al., 2000), and the viruses have a role, although quantitatively uncertain, in the global decline of amphibians (Daszak et al., 1999).
Several iridovirus-like particles were found in normal and diseased specimens of the portuguese herpetofauna (Alves de Matos, 2001). One of these was found in diseased *Triturus marmoratus* from Peneda-Gerês Natural Park, NW Portugal, in high mortality episodes of unknown aetiology (Soares et al., 2003).

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Several iridovirus-like particles were found in normal and diseased specimens of the portuguese herpetofauna (Alves de Matos, 2001). One of these was found in diseased *Triturus marmoratus* from Peneda-Gerês Natural Park, NW Portugal, in high mortality episodes of unknown aetiology (Soares et al., 2003).

Diseased *Triturus marmoratus* have been found in two lagoons widely separated in the Park. Also the disease seems to be crossing species barriers and affecting also *Triturus boscai, Alytes obstetricans* and *Rana perezi* (Soares et al., accompanying paper).
In this work we searched for virus-like particles in organs of diseased animals and present a characterization of the associated pathologic features.

MATERIALS AND METHODS

For histopathological studies, fragments of affected tissues were fixed for light microscopy with 10% buffered formalin and embedded in paraffin. Five to ten micrometer sections were stained with H+E and studied and photographed with a Leica DMLB 100S light microscope equipped with a DC300 digital camera.

Search for viruses in the diseased tissues was performed by transmission electron microscopy. Fragments of less than 1 mm^3 of the specimens were processed by standard techniques as described previously (Alves de Matos et al., 2002).

RESULTS

Infected animals displayed haemorrhagic areas showing through the mucous membranes and skin ulcerations (Soares et al., accompanying paper).

Histologic study of the haemorrhagic zones disclosed the presence of necrotic regions associated with the haemorrhages and numerous pycnotic cells (Fig. 1, Appendix III, page 81). Pycnotic cells were also found in connective tissues with no gross alterations (Fig. 2, Appendix III, page 81). Electron microscopic examination of the tissues, disclosed the presence of infected connective tissue cells (Fig. 3, Appendix III, page 81), and of large cell-free aggregates of virus particles. The virions showed pentagonal or hexagonal profiles with a diameter of 120nm between faces (Fig. 4, Appendix III, page 81).
The dermis underlying the ulcerations showed also the presence of infected connective tissue cells, probably macrophages (Fig. 3, Appendix III, page 81). The epithelial cells adjacent to the ulcerations were also infected (Figs. 5-7, Appendix III, page 81).

Infection was found in the upper layers of the epidermis in places where the basal layers were still intact (Fig. 5, Appendix III, page 81). The infected epidermal cells in various stages of differentiation often presented large perinuclear inclusions where virus morphogenesis was taking place (Fig. 6, Appendix III, page 81) and their cytoplasm was filled with a variable amount of virus particles (Fig.7, Appendix III, page 81).
Infected cells and cell debris were found in large numbers within in liver sinusoids (Fig. 8, Appendix III, page 81).

DISCUSSION

Virus-like particles found in skin ulcers and haemorrhagic areas of the diseased animals from Carris and Batateiró lagoons are morphologically similar to the *Iridoviridae*. Furthermore, their size is compatible to the members of the *Ranavirus* genus, and smaller than other known members of the vertebrate *Iridoviridae* and other unclassified iridovirus-like particles (Devauchelle et al., 1985).

The pathological features observed, namely skin ulcers and haemorrhagic areas are also compatible to known ranavirus-induced pathologies in frogs and newts (Cunningham et al., 1996; Bollinger et al., 1999), and strongly suggest that the virus may be the aetiological agent of the disease. However further evidence including experimental fulfilment of Koch's postulates is needed to establish this point.

The infection of the upper layer of the epidermis demonstrates that epithelial cells are susceptible, suggesting that these cells do not provide a protective layer to virus present in the water. This could lead to high susceptibility to infection, contributing to the high mortality observed (Soares et al, 2003). Circulating virus found in the liver sinusoids and infection of macrophagic cells show that the infection is systemic and targets the reticuloendothelial system, probably leading to immunosupression. These pathological features are found in other highly lethal virus infections like African swine fever virus (Hess, 1981).

Since the lagoons are widely separated and have no connection to each other, apart from possible human-mediated transportation, the relationships between the particles from the two lagoons it is unclear. In the absence of further virological characterization, these can not be further evaluated as well as their relationships to other known viruses (Williams, 1996).

Infection of newt populations is known in North America (Bollinger et al., 1999), but has not so far been detected elsewhere. This is the first observation of iridovirus-like viruses infecting newts in Europe. Iridoviruses are known to be geographically related (Hyatt et al., 2000). Knowledge of the virus relationships could therefore provide clues to its origin. Since iridoviruses, and in particular ranaviruses are able to cross species barriers (Moody & Owens, 1994), the presence of related, potentially highly pathogenic virus in the Peneda-Gerês National Park, poses a threat to other species, not only amphibians but also autochthonous fish species and commercial aquaculture facilities nearby. The herpetofauna constitutes a reservoir of such viruses whose monitorization has been proposed as in the context of pecuary sanitary measures (Mao et al., 1999).

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ENFERMEDADES EMERGENTES EN ANFIBIOS: LA GRAN AMENAZA

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El declive de las poblaciones de anfibios en la actualidad está fundamentalmente relacionado con la alteración y destrucción de sus hábitats, aunque en realidad existen muchas otras causas que pueden actuar de forma sinérgica. Sin embargo, mucho más reciente y preocupante es el declive de poblaciones de anfibios sin causas "aparentes", que frecuentemente además se produce en áreas bien conservadas y protegidas tales como los Parques Nacionales.

Estos recientes episodios de declive y extinción de poblaciones y especies están motivados por agentes bióticos muy diferentes de fenómenos tales como la contaminación, el calentamiento global, o el incremento de radiación ultravioleta. Así, junto con las especies introducidas (fundamentalmente peces y cangrejos), otros nuevos y mas peligrosos organismos (virus y hongos) se han incorporado a la lista de amenazas con el concepto de "enfermedad emergente" (enfermedad infecciosa de reciente aparición o cuya incidencia o rango geográfico ha aumenta recientemente drásticamente).

Los iridovirus son un tipo particular de virus que pueden producir grandes mortalidades en masa en poblaciones de anfibios, si bien todavía no puede establecerse rigurosamente su relación con el declive global de los anfibios. Los casos de mortalidades en masa por iridovirus se han producido prácticamente en todo el mundo, sin que se conozca los factores ambientales que motivan su desarrollo. Estos virus son extraordinariamente resistentes y altamente infecciosos, y en ocasiones afectan también a reptiles y peces, lo que facilita su dispersión y dificulta enormemente su erradicación.

La sintomatología de las enfermedades provocadas por iridovirus es complicada, y muchas veces se producen además infecciones bacterianas secundarias (como el síndrome de la "pata roja" producido por la proliferación de la bacteria *Aeromonas hydrophila* en animales enfermos). Los vectores de transmisión de los iridovirus son diversos, incluyendo el ser humano que los introduce en el medio mediante la suelta de peces infectados o facilita su dispersión a través del material de campo infectado.

Por otro lado, una recién descrita especie de hongo quitridio, *Batrachochytrium dendrobatidis*, se ha convertido en una de las principales causas del declive de los anfibios en todo el mundo. Hasta que en 1998 se estableció la relación entre este nuevo organismo y las mortalidades en masa de anfibios ocurridas en amplias zonas del planeta, los quitridios eran conocidos sólo como parásitos de plantas, algas, protistas e invertebrados. La característica mas importante de estos hongos es que presentan esporangios sin opérculos y zooesporas con flagelos móviles. No existen diferencias morfológicas, estructurales ni genéticas relevantes entre los quitridios de todo el mundo, por lo que parece tratarse de una única especie. Las zooesporas de estos hongos se liberan en el agua,

APPENDIX II

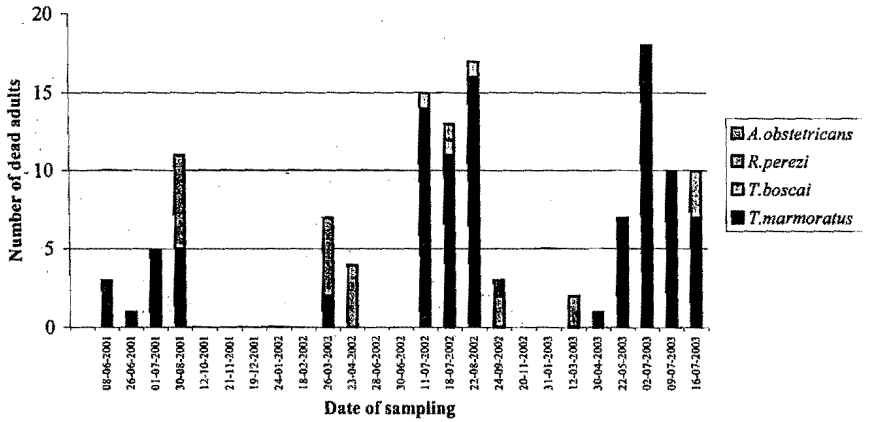


Fig. 4 - Counts of dead amphibians in Carris pond during the period of 2001 to 2003, referred to page 32.

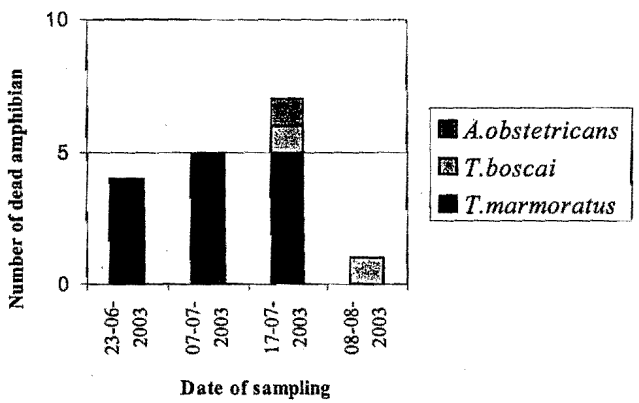
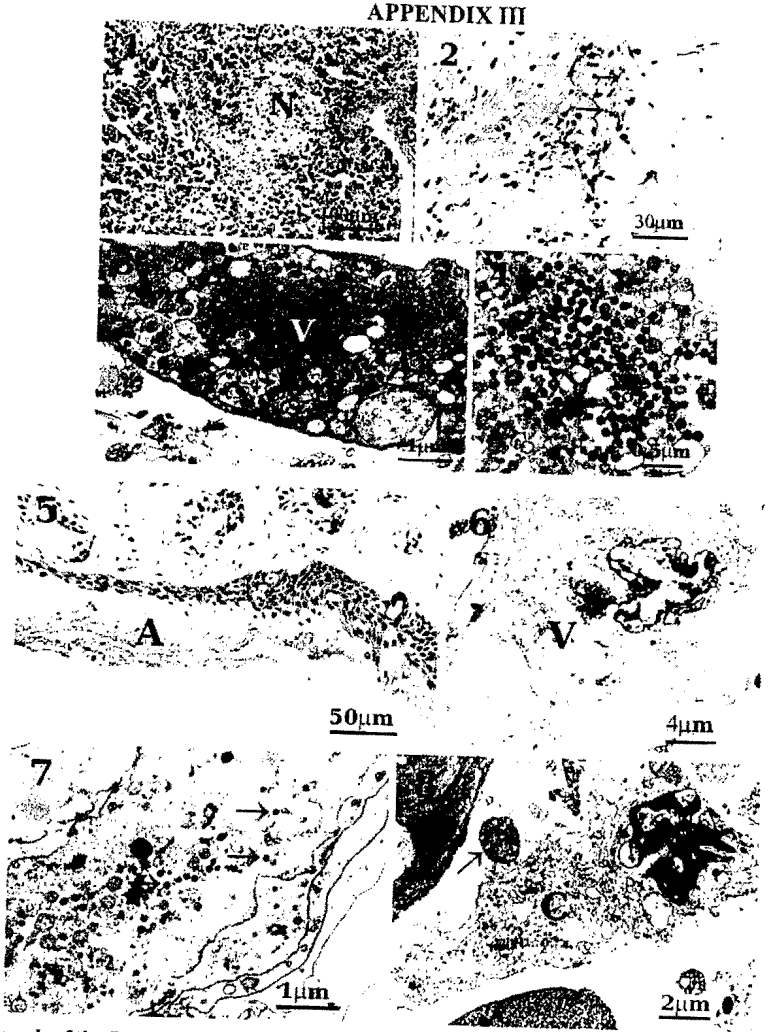


Fig.5 - Counts of dead amphibians in Batateiro pond during 2003, referred to page 40.

APPENDIX III



Legends of the figures (Referred at page 44)

Fig 1 – Necrosis (N) within hemorrhagic region.
Fig 2 – Apoptotic cells in muscle associated connective tissue (arrows).
Fig 3 – Infected macrophage in connective tissue. V – virus assembly site.
Fig 4 – Virus-like particles associated with cellular debris (arrows).
Fig 5 – Alterations of the external layers of the epidermis (A).
Fig 6 – Infected epithelial cell containing a perinuclear virus assembly site (V).
Fig. 7 – Virion accumulation in the cytoplasm of epidermal cell (arrows).
Fig. 8 – Infected cells (C) and cell debris (arrow) within hepatic sinusoids.

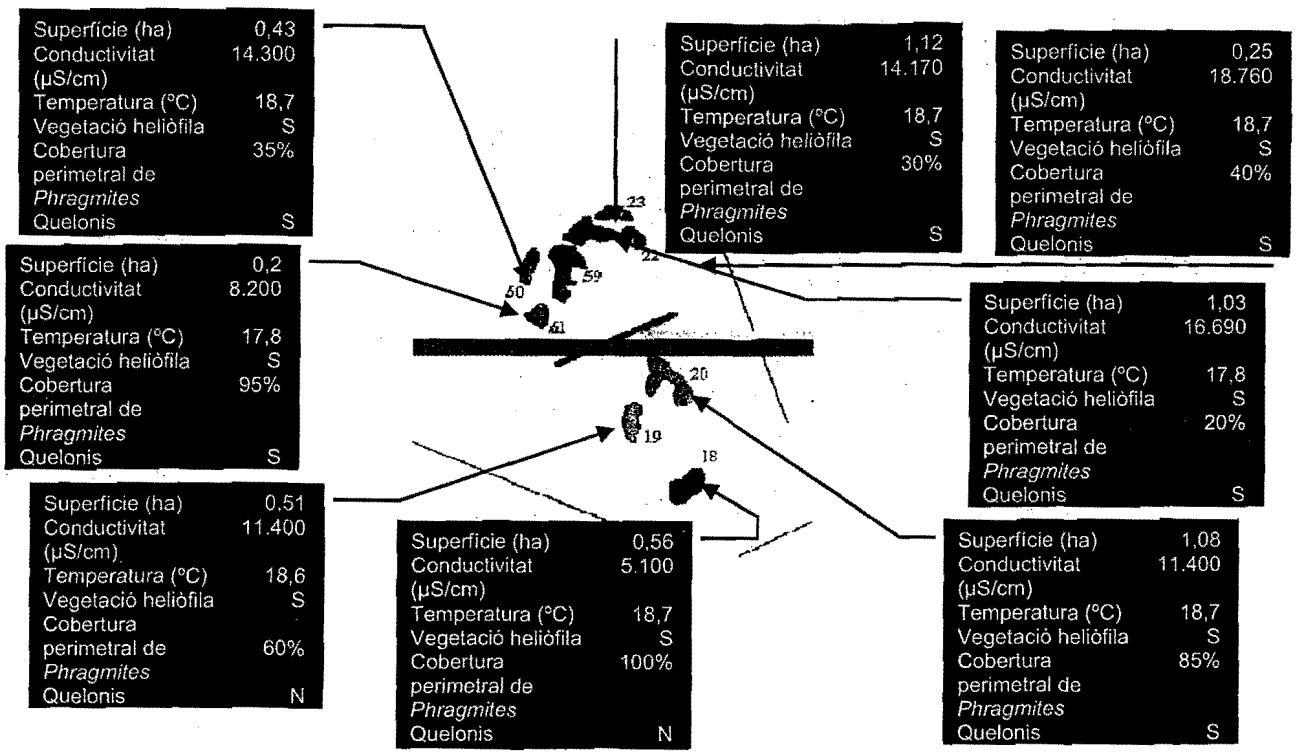


Figura 2. Caracterització fisicoquímica i biològica de cada bassa i nombre d'individus d'*Hyla meridionalis* localitzats i capturats. S'indica entre parèntesis els individus capturats durant la segona campanya. * *Gambusia affinis* ** *Gambusia affinis* i *Cyprinus carpio*. referit a la pàgina 56

APPENDIX II

Fig.1- Diseased individual of *Triturus marmoratus*, presenting cutaneous lesions on the head and forelimb referred to page 39.

Fig.2- Dead *Triturus boscai* referred to page 39.

Fig.3- Dead *Alytes obstetricans* referred to page 39.